c) optionally, a reference biological sample containing a polyclonal or monoclonal antibody recognizing the purified polypeptide or the oligomeric polypeptide of step a) (positive control); and

d) optionally, a reference biological sample that does not contain a polyclonal or monoclonal antibody recognizing the purified polypeptide or the oligomeric polypeptide of step a) (negative control).

SUPPORT FOR THE AMENDMENTS

Applicants have rewritten claims 16-24, 26-29, 31, and 33 as new Claims 56-70, to obviate the criticisms raised in the Official Action. In particular, Claim 16 has been rewritten in independent form as new Claim 56. Accordingly, support for Claim 56 can be found in Claims 1 and 9, as originally filed. Support for Claims 57-70 can be found in Claims 17-24, 26-29, 31, and 33 as originally filed.

No new matter has been added. Claims 56-70 are active in this application.

REMARKS

At the outset, Applicants wish to thank Examiner Swartz for indicating that the present claims are free of the prior art.

The objection to the specification has been obviated by appropriate amendment. As the Examiner will note, Applicants have amended the specification such that it is free of the criticisms outlined on page 3 of the Official Action.

The rejection of Claims 16-24, 26-29, 31, and 33 under 35 U.S.C. § 112, second paragraph; the rejections of Claims 21 and 22 under 35 U.S.C. § 112, second paragraph; the

rejection of Claim 23 under 35 U.S.C. § 112, second paragraph; the rejection of Claims 16, 23, 24, 26, 27, 28, 29, 31, and 33 under 35 U.S.C. § 112, second paragraph; the rejection of Claims 23, 24, 26-29, 31, and 33 under 35 U.S.C. § 112, second paragraph; and the rejection of Claims 31 under 35 U.S.C. § 112, second paragraph have been obviated by appropriate amendment. As the Examiner will also note, Applicants have rewritten the claims such that they are free of the criticisms set forth on pages 4-7, of the specification. Thus, these rejections are no longer tenable and should be withdrawn.

The rejection of Claim 24 under 35 U.S.C. § 112, second paragraph is respectfully traversed. Applicants submit that the term "additional T-epitope" as used in the present claims would be clear to one of skill in the art. In support of this assertion, the Examiner's attention is directed toward page 37, line 24, to page 38, line 23, of the specification. Clearly, in view of the present specification, the term "additional T-epitope" is definite. Accordingly, the rejection should be withdrawn.

The rejection of Claims 27-29 under 35 U.S.C. § 112, first paragraph, has been obviated by appropriate amendment. As the Examiner will note, the present claims do not recite the term "vaccine." Again, the rejection should be withdrawn.

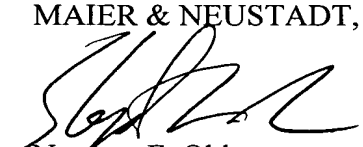
The rejection of Claims 31 and 33 under 35 U.S.C. § 112, first paragraph, is respectfully traversed. Applicants submit that the present specification provides ample instruction on how to use the method and kit of present Claims 69 and 70. In particular, the Examiner's attention is directed toward the results presented in Example 8 on page 55 of the present specification. Clearly, present Claims 69 and 70 are fully supported by the present specification.

In further support of the efficacy of the presently claimed method and kits, the Examiner's attention is directed toward Journal of Infectious Diseases, 2000:181, pp. 1850-1853 (2000), a copy of which is being submitted herewith as Exhibit A. Certainly, any doubts of the efficacy of the presently claimed method and kits are erased by the results presented in this publication. Accordingly, the rejection should be withdrawn.

Applicants submit that the application is now in condition for allowance, and early notification of such action is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.



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MARKED-UP COPY OF AMENDMENT FILED HEREWITH

IN THE SPECIFICATION

Page 1, after the title, please insert:

--This application is a 371 of International application PCT/IB98/01091 filed on July 16, 1998, which claims benefit of U.S. provisional application 60/052,631, filed on July 16, 1997.--

Page 33, line 23, to page 34, line 10, amend to read as follows:

--[Are also] Also part of the present invention are polypeptides that are homologous to the initially selected polypeptide bearing at least an epitope unit. By homologous peptide according to the present invention is meant a polypeptide containing one or several amino acid substitutions in the amino acid sequence of the initially selected polypeptide carrying an epitope unit. In the case of an amino acid substitution, one or several - consecutive or non-consecutive- amino acids are replaced by [«equivalent»] "equivalent" amino acids. The expression [«equivalent»] "equivalent" amino acid is used herein to name any amino acid that may be substituted for one of the amino acids belonging to the initial polypeptide structure without decreasing the binding properties of the corresponding peptides to the monoclonal antibody that has been used to select the parent peptide and without decreasing the immunogenic properties against the specified pathogenic microorganism. Thus, an homologous polypeptide according to the present invention has the same immunological characteristics as the parent polypeptide (for example as the polypeptide of SEQ ID NO 5)

with respect to the ability to confer [increases] increased resistance to infection with bacteria belonging to the tuberculosis complex. These equivalent [aminoacyles] amino acids may be determined either by their structural homology with the initial [aminoacyles] amino acids to be replaced, by the similarity of their net charge, and by the results of the cross-immunogenicity between the parent peptides and their modified counterparts.--

Page 54, line 15, to page 55, line 2, amend to read as follows:

--The ESAT-6 protein consists of 95 amino acids and was previously shown to be present in the [M. tuberculosis] *M. tuberculosis* ST-CF. Since *lhp* is next to esat-6, and potentially encodes a polypeptide of 100 amino acids, we investigated its eventual presence in the M. tuberculosis ST-CT. Low molecular weight ST-CF fractions were separated by preparative SDS-PAGE and submitted to systematic N-terminal sequencing. As shown in figure 11, fraction number 4 yielded a peptide sequence matching almost perfectly (14/15) with the [N-teminus] N-terminus deduced from the M. *tuberculosis lhp* gene sequence. This 10 kDa culture filtrate protein was referred to as CFP-10. To further characterize the *lhp* gene product, we over-expressed and purified recombinant CFP-10 in E. coli, in fusion with a stretch of 8 histidines. Separation of CFP-10 by SDS-PAGE indicated an apparent molecular weight of 14 kDa (Figure 12 B), [sligntly] slightly higher than the apparent molecular weight of recombinant ESAT-6 (His6) (10 kDa). The difference of size between native and recombinant CFP-10 may be [attribuable] attributable to the presence of the histidine tag. These results demonstrated that M. *tuberculosis lhp* is a gene and encodes a small polypeptide, which like ESAT-6, is found in the low-molecular weight fraction of the ST-CF. In spite of the fact that no obvious exportation signal was identified so far in the sequence of

LHP, our data suggest this protein is released extracellularly during broth cultivation of M. tuberculosis, as already observed for ESAT-6.--

Page 55, lines 16-22, amend to read as follows:

--Guinea pigs

CFP10 has been tested on BCG vaccinated, [M. avium] *M. avium* and [M. tub] *M. tuberculosis* infected and naive animals. In BCG vaccinated, [M. avium] *M. avium* infected and naive animals no DTH response was measured compared to [M. tub] *M. tuberculosis* infected [were] where a significant DTH response was observed.--

Page 55, lines 24-31, amend to read as follows:

--[Cattles] Cattle

DTH-response:

CFP10 has been tested on both [M. avium] *M. avium* and [M. bovis] *M. bovis* infected animals. In [M. avium] *M. avium* infected (ppdA positive) animals no DTH response was measured compared to [M. bovis] *M. bovis* (ppdB positive) infected [were] where a significant DTH response was observed in many of the [cattles] cattle. Further more blood cells isolated from [cattles] cattle infected with [M. bovis] *M. bovis* induced an [in vitro] *in vitro* proliferative response and release of IFN-γ after stimulation with CFP10.--

IN THE CLAIMS

Please cancel Claims 1-55, without prejudice toward the further prosecution of these claims in Continuation and/or Divisional Application. Please add the following new claims:

--56. (New) to 70. (New)--

CONCISE COMMUNICATION

Detection of Active Tuberculosis Infection by T Cell Responses to Early-Secreted Antigenic Target 6-kDa Protein and Culture Filtrate Protein 10

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The purified protein derivative (PPD) skin test has no predictive value for tuberculosis (TB) in *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)–vaccinated individuals because of cross-reactive responses to nonspecific constituents of PPD. T cell responses to early-secreted antigenic target 6-kDa protein (ESAT-6) and the newly identified culture filtrate protein 10 (CFP-10), 2 proteins specifically expressed by *M. tuberculosis* (MTB) but not by BCG strains, were evaluated. Most TB patients responded to ESAT-6 (92%) or CFP-10 (89%). A minority of BCG-vaccinated individuals responded to both ESAT-6 and CFP-10, their history being consistent with latent infection with MTB in the presence of protective immunity. No responses were found in PPD-negative controls. The sensitivity and specificity of the assay were 84% and 100%, respectively, at a cutoff of 300 pg of interferon-γ/mL. These data indicate that ESAT-6 and CFP-10 are promising antigens for highly specific immunodiagnosis of TB, even in BCG-vaccinated individuals.

Tuberculosis (TB) remains a major global public health problem. Timely diagnosis of active pulmonary cases is important for TB control, yet isolation of *Mycobacterium tuberculosis* (MTB) can take up to 6 weeks, and cultures may remain falsely negative. The sensitivity of the purified protein derivative (PPD) skin test for detection of active TB does not exceed 75%, and negative results are often associated with extensive disease [1]. In individuals vaccinated with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) or exposed to environmental mycobacteria, PPD skin testing is unreliable because of cross-reactive (false-positive) immune responses to antigens common to MTB and nonpathogenic mycobacteria [1]. A more specific diagnostic assay should preferably be based on antigens that are present exclusively in MTB but not in BCG.

Subtractive DNA hybridization of pathogenic *M. bovis* and BCG [2] and comparative DNA-microarray analysis of MTB H37Rv and BCG [3] have identified 1 region of difference, designated RD1, that was found to be present in all MTB and pathogenic *M. bovis* strains but lacking in all BCG vaccine strains and almost all environmental mycobacteria. One antigen encoded by RD1 is the early-secreted antigenic target 6-kDa protein (ESAT-6) [4, 5], which is immunodominant in mice [6]. T cell responses to ESAT-6 can discriminate between cattle infected with *M. bovis* and cattle sensitized to environmental mycobacteria [7]. In previous studies of human pulmonary TB, T cell responses to ESAT-6 were observed in approximately half of the patients [8–10]. Recently, a second RD1-encoded protein, designated culture filtrate protein 10 (CFP-10), was identified [11]. Recent results indicate that this antigen is strongly recognized by T cells [12, 13]. In the present study, we evaluated T cell responses to ESAT-6 and CFP-10 in patients with TB in comparison with various control groups without TB, including BCG-vaccinated individuals.

Subjects and Methods

Subjects. Thirty-seven TB patients aged 45.9 ± 16.1 years (mean ± SD; range, 12–70 years) were recruited at the Leiden University Medical Center in Leiden or at the Rijnland Hospital in Leiderdorp, The Netherlands. The patients originated from Western Europe ($n = 20$), Africa ($n = 8$), Asia ($n = 4$), South America ($n = 4$), or the Middle East ($n = 1$). The localization of TB was pulmonary ($n = 20$), pleural ($n = 6$), lymphatic ($n = 5$), skeletal ($n = 5$), urogenital ($n = 4$), peritoneal ($n = 3$), adrenal ($n = 1$), cerebral ($n = 1$), cutaneous ($n = 1$), or subcutaneous ($n = 1$). TB was

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The study protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center (protocol P136/97).

All subjects gave permission for blood sampling after written information was provided.

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present at ≥1 site in 9 patients. We included patients before treatment was started, as well as patients during and after treatment, to evaluate the time course of immune responses. Patients infected with human immunodeficiency virus were excluded because cell-mediated immunity to mycobacteria has been shown to be defective in the presence of low $CD4^+$ cell counts.

Twelve individuals with documented PPD conversion after contact with contagious TB, 14 BCG-vaccinated subjects (10 of whom were PPD positive), and 8 healthy, PPD-negative, non–BCG-vaccinated subjects, including medical, nursing, and technical hospital personnel and relatives of TB patients, were recruited from various sources.

Antigens. Recombinant ESAT-6 (batch p432) and CFP-10 (batches 98-2 and 99-1) were expressed in *Escherichia coli*, as described elsewhere [4, 5, 11]. MTB H37Rv sonicate was provided by Dr. D. van Soolingen (National Institute of Public Health and Environment, Bilthoven, The Netherlands). The production of short-term culture filtrate (ST-CF) has been described elsewhere [14].

Cellular stimulation assays. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. Cells were frozen in RPMI 1640 (Gibco, Paisley, Scotland) supplemented with 0.04 mM/mL glutamine, 20% fetal calf serum, and 10% dimethyl sulfoxide. For the experiments, PBMC (1.5×10^5 per well) were incubated in round-bottom microtiter wells in the presence or absence of antigen in 200 μL of Iscoves modified Dulbecco's medium (Gibco), supplemented with 10% pooled human AB serum, 40 U/mL penicillin, and 40 μg/mL streptomycin in triplicate at 37°C in humidified air containing 5% CO_2. The final concentrations of the antigens used were as follows: ESAT-6, 1 and 10 μg/mL; CFP-10, 0.5 and 5 μg/mL; MTB sonicate and ST-CF, 0.1 and 1 μg/mL each. Supernatants for interferon-γ (IFN-γ) determinations, as the readout of T cell activation, were collected at days 3 and 6 (50 μL/well) and pooled per triplicate. Responses at day 6 were optimal and were used for the analysis.

IFN-γ production. IFN-γ was measured with a standard ELISA technique (U-CyTech, Utrecht, The Netherlands). The detection limit of the assay was 20 pg of IFN-γ/mL. IFN-γ values in unstimulated cultures were typically undetectable, except in 15 (11%) of 136 unstimulated triplicates with a median concentration of 62 pg/mL. Detectable values were subtracted from the value in stimulated cultures.

Statistical analysis. Differences between responses were tested with the nonparametric Kruskal-Wallis and Mann-Whitney tests. Correlation of individual responses to different antigens was analyzed by Spearman correlation. Receiver-operator characteristic (ROC) curves were constructed to describe the relation between the sensitivity and specificity at varying cutoff levels. All statistical analyses were 2 sided, and P values <.05 were considered statistically significant.

Results

Responses to MTB sonicate and ST-CF. T cell responses to MTB sonicate in TB patients (figure 1*A*) were significantly higher than those in PPD-negative controls without BCG vaccination but were not different from those in BCG-vaccinated or PPD-converted individuals. T cell responses to ST-CF of TB patients (figure 1*B*) were significantly higher than the responses of the other groups.

TB patients and control groups respond differently to ESAT-6 and CFP-10. Responses to ESAT-6 (figure 1*C*) and CFP-10 (figure 1*D*) differed highly significantly between the study groups ($P < .0001$ for comparison of all groups). Individual responses to ESAT-6 and CFP-10 were highly correlated ($r = .79$; 95% confidence interval, 0.67–0.86; $P < .0001$ [data not shown]). Most patients given a diagnosis of TB produced IFN-γ in response to ESAT-6 (34/37 [92%]), CFP10 (33/37 [89%]), or both (32/37 [86%]), and 35/37 (95%) responded to ≥1 of both antigens. Responses to ESAT-6 and CFP-10 were independent of age and sex and were similar in patients with pulmonary or extrapulmonary TB and in those with TB at ≥1 site. The geometric mean ± SD of IFN-γ responses was lower at the time of diagnosis than during or after treatment (639 ± 2550 vs. 1228 ± 4407 pg/mL for ESAT-6, $P = .26$; 326 ± 938 vs. 990 ± 6104 pg/mL for CFP-10, $P = .04$), but the range of responses was similar.

Nine of 12 individuals with documented PPD conversion after contact with contagious TB responded to ESAT-6; 4 of those 9 individuals also responded to CFP-10. Five (36%) of 14 BCG-vaccinated persons responded to ESAT-6, and 5 (36%) of 14 responded to CFP10, with concordant responses in 4, all of whom were PPD positive and had a history compatible with past exposure to MTB. None of them had ever had clinical signs suggestive of TB. None of the BCG-vaccinated individuals who were nonresponsive to ESAT-6 and CFP-10 had recognized exposure to MTB, but all had been vaccinated with BCG because they were in high-risk jobs. None of the PPD-negative, non–BCG-vaccinated controls responded to ESAT-6 or CFP-10.

Cutoff level to define a positive test result. ROC curves were constructed from the responses to MTB sonicate (figure 2*A*), ST-CF (figure 2*B*), and the individual maximum of responses to ESAT-6 and CFP-10 (figure 2*C*), comparing all 37 TB patients with BCG-vaccinated and PPD-negative, non–BCG-vaccinated controls, taken together as a group without TB. At a cutoff level of 300 pg/mL, the sensitivity of the individual maximum of the responses to ESAT-6 and CFP-10 was 84%, and the specificity was 100%. ROC analysis limited to TB patients who were tested before anti-TB treatment was initiated ($n = 13$) resulted in similar ROC curves as those based on all 37 TB patients (figure 2*D* through 2*F*), indicating that the postinfection interval was not a major determinant of the overall test performance.

Discussion

In the present study, T cell responses to ESAT-6 and the newly identified RD1-encoded antigen CFP-10 are shown to

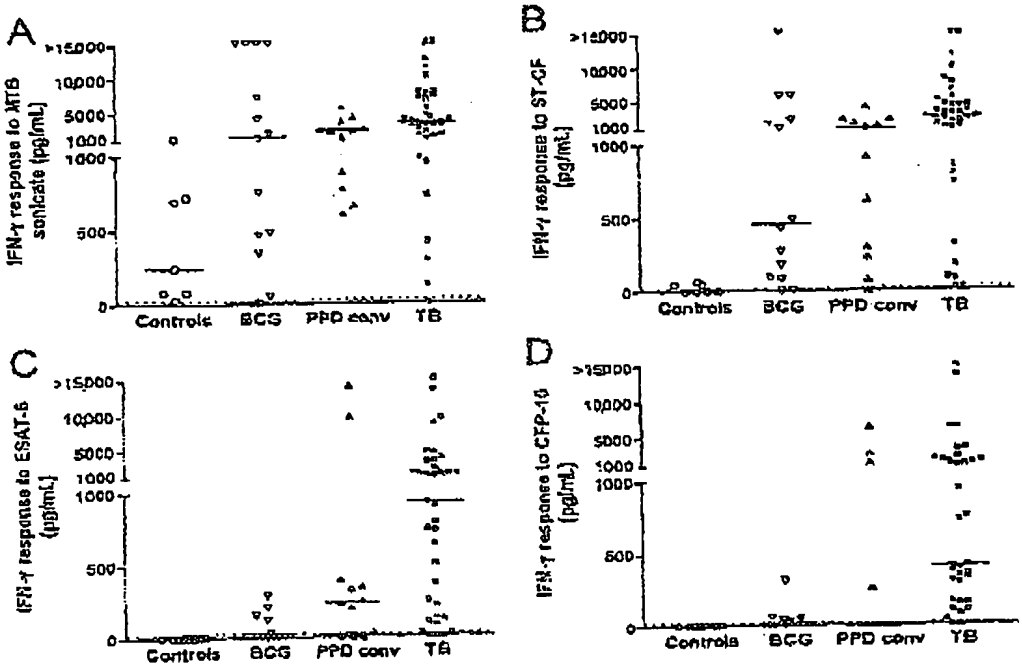

Figure 1. T cell responses of patients with tuberculosis (TB; ■) to *Mycobacterium tuberculosis* (MTB) sonicate *(A)* were significantly higher than those of controls negative for purified protein derivative (PPD) and without *M. bovis* bacillus Calmette-Guérin (BCG) vaccination (○; $P = .0005$) but not different from those of BCG-vaccinated (▽) or PPD-converted (▲) individuals. T cell responses of TB patients to short-term culture filtrate (ST-CF) *(B)* were significantly higher than those of each of the other groups ($P = .0002$, $P = .01$, and $P = .007$ for the comparisons with PPD-negative, BCG-nonvaccinated; BCG-vaccinated; and PPD-converted subjects, respectively). Interferon-γ (IFN-γ) production in response to early-secreted antigenic target 6-kDa protein (ESAT-6) *(C)* and culture filtrate protein–10 (CFP-10) *(D)* was significantly higher in TB patients than in BCG-vaccinated and PPD-negative, non–BCG-vaccinated controls ($P < .0001$ for all comparisons). Comparison of TB patients and individuals with documented PPD conversion after exposure to MTB demonstrated significant differences in responses to CFP-10 ($P = .02$) and differences of borderline significance in responses to ESAT-6 ($P = .07$). Statistical comparison between groups was performed using the Mann-Whitney *U* test. Short lines represent geometric mean responses for the groups. Dotted lines represent detection limit of the IFN-γ ELISA (20 pg/mL).

be highly sensitive and specific for discrimination between patients with TB disease and noninfected individuals and to be significantly more specific than responses to the complex antigens MTB sonicate and ST-CF. Most patients with active or treated TB in our study responded to the specific antigens ESAT-6 or CFP-10 or both. The higher sensitivity of responses to ESAT-6 than that found in previous studies [8–10] could be related to differences in test conditions. Our results strongly support larger prospective studies to establish the actual predictive value of T cell responses to ESAT-6 and CFP-10 in patients suspected to have TB disease, and we recently started such a study. Moreover, ongoing follow-up studies of healthy, untreated, PPD-positive TB contacts indicate that responses to ESAT-6 are associated with the risk of developing active TB (authors' unpublished observations). Factors contributing to the observed variability of responses to ESAT-6 and CFP10 in the TB patients could include mycobacterial load, antigen expression level of MTB strains, and genetically based characteristics of antigen processing and immune responsiveness in the host, including cytokine secretion profiles or polymorphisms of HLA or cytokine receptors.

The majority of the PPD-converted individuals and several of the BCG-vaccinated subjects in our study responded to ESAT-6, and some also responded to CFP-10, presumably as a result of latent infection with MTB. Latent infection with MTB is 10 times more frequent than active TB, with global prevalence rates estimated to amount to one-third of the world's population. Subclinical or latent infection with MTB was the presumed cause of T cell responses to ESAT-6 observed in many Ethiopian control subjects in a previous study [8]. Today, half

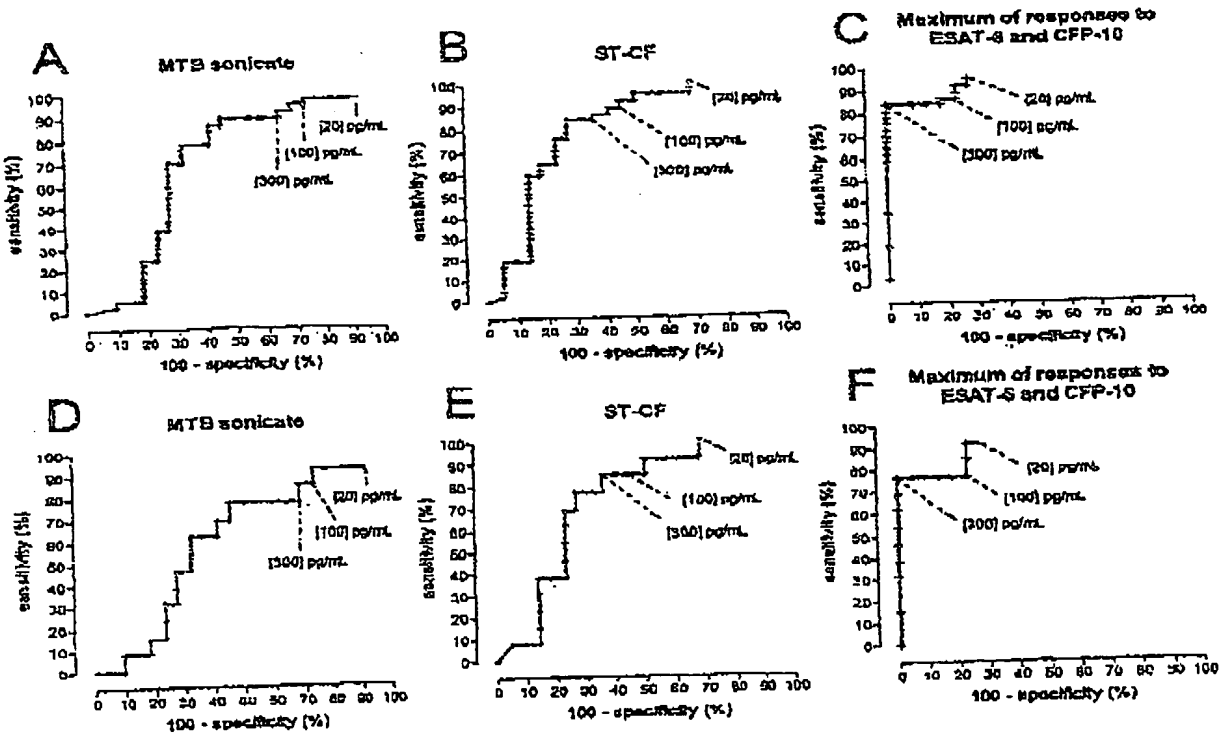

Figure 2. Receiver-operator characteristic (ROC) curves were constructed from responses to *Mycobacterium tuberculosis* (MTB) sonicate *(A)*, short-term culture filtrate (ST-CF) *(B)*, and the individual maxima of responses to early-secreted antigenic target 6-kDa protein (ESAT-6) and culture filtrate protein–10 (CFP-10) *(C)* in 37 tuberculosis (TB) patients and in 22 control subjects (14 *M. bovis* bacillus Calmette-Guérin [BCG]–vaccinated and 8 PPD-negative, non–BCG-vaccinated individuals). Several potential cutoff levels are indicated between brackets. Including only those TB patients from whom samples were taken before anti-TB chemotherapy had been instituted ($n = 13$) yielded ROC curves *(D–F)* similar to those including all 37 patients. The ROC curves end at a cutoff value equal to the detection limit of the ELISA (20 pg/mL), because interferon-γ production was below the detection limit in a number of subjects.

of all TB patients in Western countries originate from areas with highly endemic TB where BCG vaccination is routine practice. It must therefore be expected that latent infection with MTB, accompanied by T cell responses to MTB-specific antigens, can occur in relevant control groups.

The PPD skin test is technically simple, can be produced at low cost, and is more or less reproducible if performed by skilled personnel [1]. Yet the PPD skin test lacks specificity in BCG-vaccinated individuals, as was confirmed in the present study, in which responses to MTB sonicate were similar and those to ST-CF were only moderately different in TB patients and BCG-vaccinated individuals. Skin tests with PPDs derived from non-tuberculous mycobacteria were of variable use for discrimination between TB and infection with atypical mycobacteria [15]. The requirements for an in vitro T cell assay are more complex and include highly purified MTB-specific antigens and facilities for cell-culture and cytokine assays, limiting its use as a routine diagnostic tool. However, antigens that appear specific in an in vitro T cell assay could be evaluated in a skin test. This preferably would be a test based on a combination of several specific antigens; in animal models of TB, the sensitivity of a skin test based on recombinant antigens was reported to increase with the number of antigens [16].

The functions of ESAT-6 and CFP-10 remain to be elucidated, but conceivably they contribute to virulence, because RD1 was deleted early during the process of attenuation of pathogenic *M. bovis* to essentially avirulent BCG [3]. Immunization of mice with DNA encoding for ESAT-6 conferred moderate protection against airborne experimental TB, although substantially less than that achieved by BCG vaccination [17]. ESAT-6 and CFP-10 probably have related functions, because both genes are part of the same operon, and